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Human bronchus-mediated mutagenesis of mammalian cells by carcinogenic polynuclear aromatic hydrocarbons

(carcinogen metabolism/benzo[*a*]pyrene/ouabain/Chinese hamster cells)

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ABSTRACT **Cultured human bronchial explants activated benzo[*a*]pyrene (Bz*a*P) into electrophilic metabolites that bind to DNA in bronchial epithelial cells. Promutagenic and mutagenic metabolites of Bz*a*P were also released into the culture medium. An increase in mutation frequency for ouabain resistance was found in Chinese hamster V-79 cells when they were cocultivated with bronchial explants in the presence of Bz*a*P. The proximate carcinogenic form of Bz*a*P, the 7,8-diol [(±)-*r*7,*t*8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene], was 5-fold more potent as a promutagen than the parent compound. Neither Bz*a*P nor the 7,8-diol increased the mutation frequency in V-79 cells when they were cultured without bronchial explants. The mutation frequency was directly related to the binding levels of Bz*a*P to bronchial DNA and the concentration of either Bz*a*P or the 7,8-diol in the medium.**

Cell-mediated mutagenesis systems have been described by Huberman and Sachs (1) and by Newbold *et al.* (2). The indicator cells for detecting mutagenicity, Chinese hamster V-79 (3), do not effectively metabolize chemical procarcinogens (promutagens) including benzo[*a*]pyrene (Bz*a*P) and its proximate carcinogenic form (±)*r*7,*t*8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (7,8-diol) and they are not mutated by these compounds (1, 2, 4). However, if V-79 cells are cocultivated with cells that are capable of metabolically activating Bz*a*P (e.g., Syrian golden hamster embryo cells), then an increase in mutation frequency in the V-79 cells can be detected. One advantage of this approach is that intact cells instead of microsomal preparations are used to activate the promutagen; the metabolism of Bz*a*P within intact cells has been shown to differ from that found in microsomes (5).

We have modified these cell-mediated mutagenesis systems by cocultivating V-79 cells with human bronchial cells in tissue explants. Explant cultures maintain the architecture of the tissue, the epithelial/stromal relationships, and the differentiated state of epithelial cells (6). Using this human tissue-mediated mutagenesis system, we found that cultured human bronchus activates both Bz*a*P and 7,8-diol into a mutagenic metabolite(s). This approach makes it possible to better define metabolic activation pathways of chemical carcinogens in human target tissues. The human tissue-mediated mutagenesis system may also be useful both in evaluating the hazards of environmental chemicals to humans and in comparing activation of chemical carcinogens in target tissues from humans and from animal models of human carcinogenesis.

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MATERIALS AND METHODS

Chemicals. [*G*-^{3}H]Bz*a*P (8.3 Ci/mmol) was purchased from Amersham/Searle Corp., Arlington Heights, IL. Thirty micrograms of Bz*a*P (National Cancer Institute Chemical Carcinogen Repository, Bethesda, MD) was mixed with 320 μCi of [^{3}H]Bz*a*P in 200 μl of dimethyl sulfoxide (Pierce Chemical Co., Rockford, IL). This solution was either added directly to the culture medium or diluted further with dimethyl sulfoxide prior to addition. 7,8-Diol was obtained from S. Yang (Chemistry Branch, National Cancer Institute) and was also dissolved in dimethyl sulfoxide before use. Benzo[*e*]pyrene (Bz*e*P) was obtained from the Chemical Carcinogen Repository. Ouabain was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Cultures. Cloned Chinese hamster V-79-4 cells were kindly supplied by E. Huberman (Oak Ridge National Laboratory, Oak Ridge, TN) and were grown in Dulbecco's modified Eagle's medium (catalog no. 196G, Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Irvine Scientific Co., Irvine, CA), penicillin (100 units/ml), streptomycin (100 μg/ml), and glutamine (2 mM). The concentration of K^+ was 2.9 mM. The cultures were dispersed with 0.05% trypsin/EDTA solution (Grand Island Biological Co.) and were subcultured twice a week at a dilution of 1:20 and incubated at 37° with 10% CO_2 in air. The initial experiments (patients 146, 148, 154, and 157B) were conducted with V-79-4 cells; these were recloned to produce clone V-79-4A which was used for the remainder of the studies.

Explant Cultures. Human bronchial specimens were obtained at the time of operation (patients 146, 157B, 161, 169, 169A, 174, 179, 181, and 182B) or immediate autopsy (7) (patients 148, 154, 163, 165, 176, 177, and 182) (Table 1). Explants (1 × 1 cm) of grossly normal-appearing bronchi from each patient were cultured in a chemically defined medium, CMRL-1066 (Grand Island Biological Co.) [containing hydrocortisone hemisuccinate, 0.1 μg/ml (The Upjohn Co., Kalamazoo, MI); crystalline bovine insulin, 1 μg/ml (Eli Lilly & Co., Indianapolis, IN); β-retinyl acetate, 0.1 μg/ml (Hoffmann-LaRoche Inc., Nutley, NJ); and penicillin G, 100 units/ml, and streptomycin, 100 μg/ml (Grand Island Biological Co.)], on a rocker platform in an atmosphere of 50% O_2/45% N_2/5% CO_2 as described (8, 9). To minimize the effect of exogenous agents on the level of aryl hydrocarbon hydroxylase (AHH) prior to culture, the explants were maintained for 7 days before exposure to either Bz*a*P or 7,8-diol.

Abbreviations: Bz*a*P, benzo[*a*]pyrene; Bz*e*P, benzo[*e*]pyrene; 7,8-diol, (±)*r*7,*t*8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; AHH, aryl hydrocarbon hydroxylase; O^r, ouabain-resistant.

Table 1. Source of bronchial specimens

Patient	Age; sex	Diagnosis
148	79; M	Traumatic head injury
157B	62; M	Squamous cell carcinoma, lung
154	33; M	Traumatic head injury
161	60; M	Adenocarcinoma, lung
163	23; M	Traumatic head injury
165	16; M	Traumatic head injury
169	66; M	Combined adenoepidermoid carcinoma, lung
169A	64; M	Carcinoid, lung
174	54; M	Adenocarcinoma, lung
176	48; M	Traumatic head injury
177	63; M	Traumatic head injury
178	68; M	Combined adenoepidermoid carcinoma, lung
181	50; F	Adenocarcinoma, lung
182	28; F	Traumatic head injury
182B	51; M	Carcinoid, lung

Mutagenesis in V-79 Cells Cocultivated with Human Bronchus. The protocol for bronchus mediated-mutagenesis is shown in Fig. 1. At 6 hr before addition of medium containing either B*za*P or 7,8-diol and bronchial explants (five to seven per dish), 2.5×10^5 V-79 cells were seeded in 60-mm tissue culture dishes (Costar, Cambridge, MA) containing 3 ml of culture medium. During the next 2 days the culture medium was removed at 9 a.m. and 6 p.m. for determinations of B*za*P metabolites by high-pressure liquid chromatography and of AHH activity, and 2.5 ml of fresh carcinogen-containing medium was added. At 48 hr after cocultivation, the explants were removed and the V-79 cells were dispersed with trypsin/EDTA solution and plated in culture medium without carcinogen at either 100 cells per 60-mm dish (5 dishes) for determination of cell survival (cloning efficiency) or 10^5 cells per 60-mm dish (10–30 dishes) for determination of ouabain-resistant (O^r) colonies. Cell counts were made with a Coulter counter, model ZB_1 (Coulter Electronics, Hialeah, FL). Cloning efficiency was determined after 7 days by dividing the number of stained colonies (>1 mm in diameter) by the total number of cells plated. After 48 hr, 4 ml of medium containing 1 mM ouabain was added to the dishes for the mutagenesis assay. The colonies were fixed with methanol and stained with Giemsa 12–14 days later. The majority of the mutagenesis experiments were done in duplicate. The variation in the results between duplicate experiments was less than 20%. One bronchial explant per experimental variable was examined by high-resolution light microscopy (9), a technique used to monitor the viability of the epithelium. All experiments were conducted under gold-colored lights.

Determination of ^{86}Rb Uptake. To measure Na^+,K^+-ATPase function (10), 8×10^3 V-79 cells were plated in 16-mm tissue culture dishes (Flow Laboratories, Rockville, MD) with culture medium and incubated at 37° for 72 hr. The medium was then replaced with fresh culture medium containing 1 mM ouabain. Three hours later, ^{86}Rb (2.6 mCi/mg; New England Nuclear, Boston, MA) was added at 1 μCi/ml for 20, 40, and 60 min. The uptake of ^{86}Rb as a function of time was assayed by removing the medium, washing the dishes twice with Dulbecco's phosphate-buffered saline, and lysing the cells with 2 ml of distilled water. The radioactivity in the lysate was measured in a gamma ray counter.

Separation of Ethyl Acetate-Extractable Metabolites of B*za*P by High-Pressure Liquid Chromatography. The metabolites were extracted from the culture medium with 5 volumes of ethyl acetate as described (11). Metabolites were separated with a Spectro Physics B-3500 high-pressure liquid chromatograph (Santa Clara, CA) fitted with a 1-m octadecyltrimethoxysilane-permaphase column. Elution was by a 30–70% methanol/water linear gradient (gradient sweep time, 30 min). Authentic ^{14}C-labeled metabolites were included in the extract as internal standards. Radioactivity measurements were made in a Searle mark 3 scintillation counter (Amersham/Searle, Arlington Heights, IL) with Aquasol (New England Nuclear) as the scintillation cocktail.

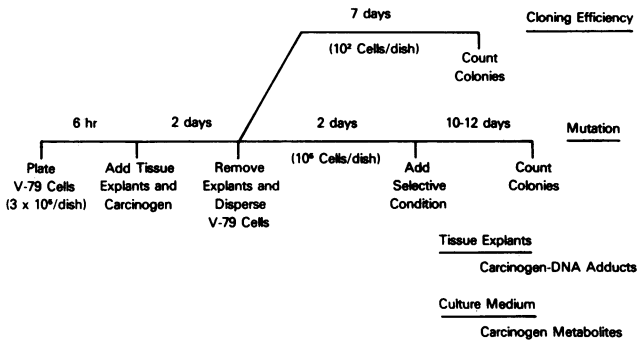

FIG. 1. Protocol for human bronchus-mediated mutagenesis assay including determination of cytotoxicity, mutation frequency, carcinogen–DNA binding levels, and carcinogen metabolites. The selective condition was 1 mM ouabain.

Determination of AHH Activity. The activity of AHH was determined by measuring the formation of ^3H_2O released into the culture medium by using a modification (12) of the method of Hayakawa and Udenfriend (13).

Binding of B*za*P to Bronchial DNA. The bronchial mucosa was scraped from the explants after cocultivation and dissolved in buffer (pH 7.5, 10 mM Tris·HCl/10 mM EDTA/10 mM NaCl/0.5% sodium dodecyl sulfate). Proteinase K (Beckman, Palo Alto, CA) was added to the solution to make a final concentration of 50 μg/ml. The solution was incubated at 37° for 2–3 hr and then extracted with an equal volume of 88% phenol. The DNA in the solution was further purified by ethanol precipitation, RNase treatment, Pronase treatment, and CsCl density gradient centrifugation and the radioactivity bound to DNA was determined as described (9).

RESULTS

Human Bronchus-Mediated Mutagenesis of V-79 Cells. Our initial efforts were directed toward defining the human tissue-mediated mutagenesis system. The optimal expression time—i.e., the time between exposure to the chemical carcinogen and the addition of the selective agent—for isolating O^r mutants was 48 hr (Fig. 2). Reconstruction experiments were done to determine the efficiency of recovering O^r mutants in this experimental system. In the absence of wild-type V-79-4A cells, the cloning efficiencies of O^r mutants with expression times of 24, 48, and 72 hr were 88%, 92%, and 91%, respectively. However, in the presence of 10^5 wild-type cells per dish, the recovery of the added O^r mutants varied with expression time (70% recovery at 24 hr, 80% at 48 hr, and 0% at 72 hr). When, after an expression time of 72 hr, the mixed cultures containing the added O^r mutants were dispersed and reseeded at 10^5 cells per dish and then 1 mM ouabain-containing medium was added 48 hr later, the recovery of the added O^r mutants was once again 80%.

When V-79 cells were cocultivated with bronchial explants and either B*za*P or 7,8-diol, an increase in O^r mutations was

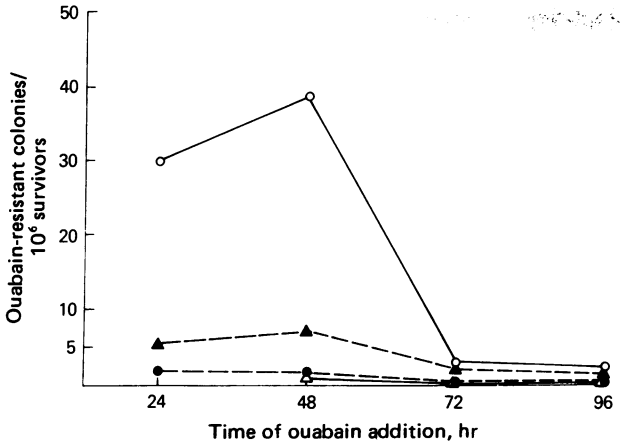

FIG. 2. Expression time for the induction of O^r mutants in V-79 cells. Ouabain (1 mM) was added to the culture medium at different times after exposure to carcinogen and replating of the cells; the colonies were stained for counting after 2 weeks of incubation at 37°. The mutation frequencies were corrected for V-79 cell survivals. Cells were from patient 154. ○, 7,8-Diol (1 μg/ml) with bronchial explants; ●, 7,8-diol (1 μg/ml) without explants; ▲, BzaP (1 μg/ml) with bronchial explants; △, BzaP (1 μg/ml) without bronchial explants.

observed. This increase was proportional to the amount of bronchial tissue added with culture medium containing 7,8-diol (1 μg/ml); cocultivation of V-79 cells with bronchial explants in culture medium free of either BzaP or 7,8-diol did not increase the O^r mutation frequency (unpublished data).

The mutation frequency for O^r in the complete system was also dependent on the concentration of either BzaP or 7,8-diol in the medium (Table 2; patients 146 and 157B). An increase in concentration of 7,8-diol from 0.1 to 1.0 μg/ml enhanced the mutation frequency approximately 5-fold. The dose-dependent effects of BzaP were less marked. 7,8-Diol induced a 4- to 8-fold higher mutation frequency than did BzaP (patients 146, 148, and 154). Compared to BzaP, the noncarcinogenic analog BzeP did not increase O^r mutation frequency (patient 161).

Characterization of Ouabain-Resistant Mutants. Colonies selected by their resistance to 1 mM ouabain were cloned and grown in the absence of the selective agent for two passages (at least 12 population doublings) before their growth and cloning efficiency in medium containing ouabain were tested. The cloning efficiency of the O^r cells in growth medium without ouabain ranged from 60 to 85%. All 12 isolated clones grew in 1 mM ouabain medium and had a cloning efficiency 50–80% of that in control medium. Similar results were obtained with four clones of O^r mutants grown for 1 month in ouabain-free medium (approximately 60 population doublings). In general, the cells formed smaller colonies in the selective medium than in control medium.

Table 2. Human bronchus-mediated mutagenesis by BzaP, BzeP, or 7,8-diol

Cell source, patient no.	Agent	Dose, μg/ml	O^r mutants per 10^6 survivors* Control	Experimental
146	7,8-Diol	0.1	1	17 (7)
	7,8-Diol	1.0	2	84 (40)
	BzaP	1.0	4	26 (11)
157B	BzaP	0.2	1	2 (1)
	BzaP	1.0	2	3 (2)
	BzaP	2.0	2	7 (4)
148	BzaP	1.0	6	14 (7)
	7,8-Diol	1.0	1	52 (33)
154	BzaP	1.0	2	7 (5)
	7,8-Diol	1.0	3	37 (40)
161	BzeP	1.0	1	1 (1)
	BzaP	1.0	2	8 (6)

* Control (V-79-4 cells incubated with 7,8-diol, BzeP, or BzaP) and experimental (V-79-4 cells incubated with 7,8-diol, BzeP, or BzaP and with bronchial explants) groups were studied in duplicate (10–14 plates each). The variation between duplicates was less than 20%. The results in parentheses were calculated per 10 μg of human bronchial DNA.

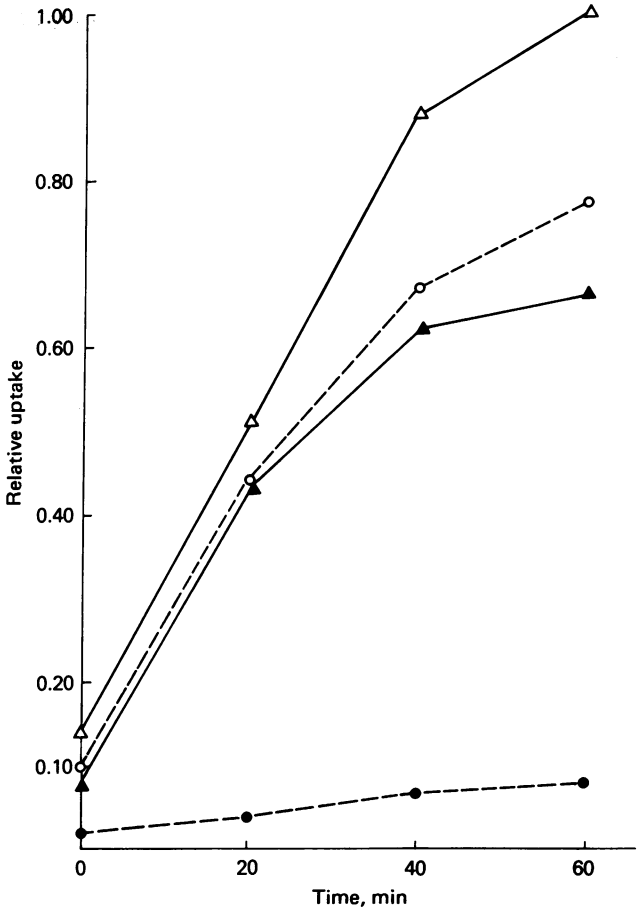

FIG. 3. Ouabain sensitivity of wild-type (○,●) and O^r (△,▲) V-79 cells. Uptake of ^{86}Rb at 37° either with (●,▲) or without (○,△) 1 mM ouabain was measured in wild-type and O^r cells as a function of time.

The Na^+,K^+-dependent ATPase is known to transport Rb^+ and K^+ into cells by the same mechanism (10). Because it has a longer half-life than ^{43}K, ^{86}Rb was used to determine altered Na^+,K^+-ATPase function in the mutants. Six O^r clones were tested for ^{86}Rb uptake, and similar results were obtained. The results from one of the experiments are illustrated in Fig. 3. Both wild-type and O^r mutants incorporated ^{86}Rb almost linearly for 60 min at 37° in medium without ouabain. The uptake of ^{86}Rb by wild-type cells was markedly inhibited by 1 mM ouabain but O^r cells were less sensitive (5–30% inhibition).

Mutation Frequency in V-79 Cells and Metabolism of BzaP in Bronchial Explants. The metabolism of BzaP was studied by measuring the levels of BzaP bound to bronchial DNA, the activity of AHH, and the profile of BzaP metabolites released into the culture medium. V-79 cells alone metabolized BzaP to only a small extent (Fig. 4). The two major peaks contained quinones which are nonenzymatic photodecomposition products and atmospheric oxidation products of BzaP. The

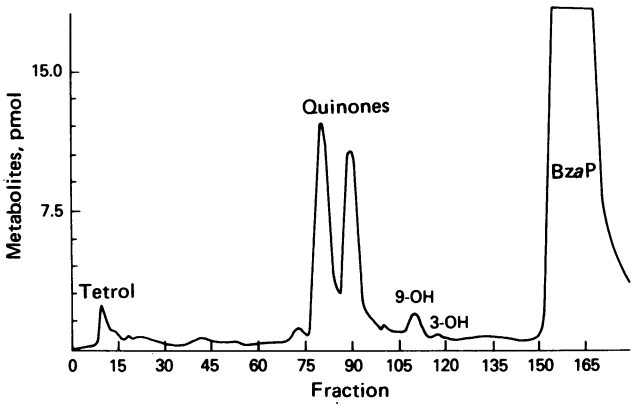

FIG. 4. Profile of metabolites of BzaP found in the culture medium of V-79 cells. Metabolites were extracted with ethyl acetate and separated by high-pressure liquid chromatography (11). Quinones are photodecomposition products of BzaP.

small peak in fractions 10–15 contained 0.2% of the total BzaP and it migrated with the tetrols of BzaP. This radioactivity peak has not been further characterized. In contrast to V-79 cells alone, cocultivation with human bronchial explants led to a more complex profile of BzaP metabolites (Fig. 5). This profile is similar to that found with cultured human bronchi alone (ref. 12; unpublished data). The 7,8-diol of BzaP was formed as well as metabolites that cochromatographed with tetrols and triols of BzaP. The small amount of quinones is consistent with the finding that these photodecomposition products are further metabolized to water-soluble conjugates (14). More than 50% of the BzaP in medium containing five to seven explants was metabolized after 12–15 hr of cocultivation (unpublished data).

The O^r mutation frequency, binding levels of BzaP to bronchial DNA, and activity of AHH are shown in Table 3. In the 13 samples of bronchial cells studied to date with V-79-4A cells, a moderate to good relationship between mutation frequency and binding levels to DNA ($r = 0.73$; $P < 0.01$) and no relationship between mutation frequency and activity of AHH ($r = 0.20$; $P > 0.1$) were found. The O^r mutation frequency in V-79-4A cells cocultivated with bronchial explants was significantly higher than that found in control cells as analyzed by parametric (Student) and nonparametric (Wilcoxon signed rank) statistical tests (15).

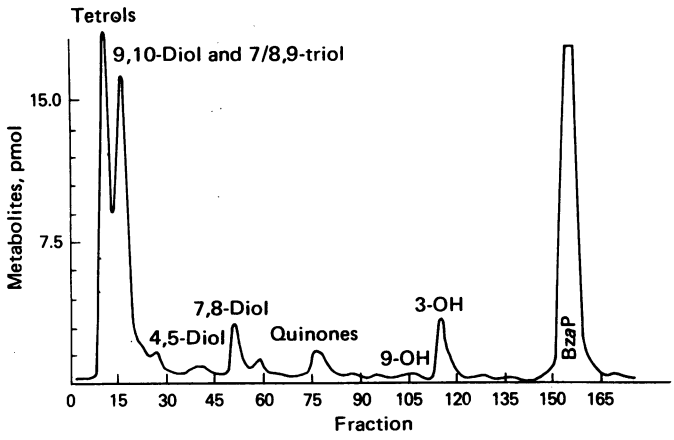

FIG. 5. Profile of metabolites with BzaP found in the culture medium of V-79 cells cocultivated with human bronchial explants (six explants per dish; patient 146). Metabolites were extracted with ethyl acetate and separated by high-pressure liquid chromatography (11).

Table 3. Metabolism of BzaP in cultured human bronchus and mutation frequency in V-79-4A cells

Cell source, patient no.	O^r mutants/10^6 survivors* Control	Experimental	BzaP binding to bronchial DNA†	AHH‡
161	2	8 (6)	162	44
163	1	3 (2)	105	18
165	1	5 (3)	142	17
169	2	4 (1)	73	64
169A	1	2 (1)	86	23
174	<1	7 (4)	104	25
176	<1	9 (4)	103	25
177	<1	8 (2)	164	6
178	<1	9 (3)	205	19
179	1	4 (5)	146	39
181	1	3 (2)	50	20
182	1	15 (9)	235	30
182B	<1	4 (6)	240	40
Mean ± SEM	1.1 ± 0.1	6.2 ± 1.0§		

* Control was V-79-4A cells incubated with BzaP (1 μg/ml) and without human bronchial explants; experimental was V-79-4A cells incubated with both BzaP and human bronchial explants. Experimental groups were usually studied in duplicate (10–12 plates each). The variation between the duplicates was less than 20%.

† pmol of BzaP bound per 10 mg of bronchial DNA.

‡ Activity of aryl hydrocarbon hydroxylase, shown as dpm/ml of culture medium per hr/10 μg of bronchial DNA.

§ For difference from control, $P < 0.001$. All values <1 (no O^r mutants in 20–30 plates) were calculated as being 1 and evaluated by the Student t and the Wilcoxon signed rank test (15).

DISCUSSION

Explants of human bronchi metabolize BzaP and release a metabolite(s) of BzaP that is mutagenic in V-79 cells. The proximate carcinogenic form of BzaP, 7,8-diol, was approximately 5-fold more potent as a promutagen than was BzaP (Table 2). Binding levels of 7,8-diol to DNA in bronchial explants were also 5- to 20-fold greater than those found with BzaP (16). In the 13 samples of bronchial cells studied with clone V-79-4A, a positive correlation between binding levels of BzaP to DNA in the cultured human bronchi and the induction of O^r mutants in V-79-4A cells was found (Table 3). No correlation was found between O^r mutation frequency and AHH activity [radiometric assay (13)], which may reflect the difficulty in correlating AHH activity with the consequence of the multistep pathway of metabolic activation for BzaP (i.e., formation of a carcinogen–DNA adduct). The between-individual variation in mutation frequency was 9-fold and the variation in binding levels of BzaP to DNA in the bronchial explants was 5-fold. Additional patients must be studied to determine if the variation in mutation frequency will eventually be as high (i.e., 75-fold) as the variation found in levels of BzaP bound to DNA in cultured bronchi from a large number of patients (17).

When the metabolic rate of BzaP was estimated by measuring the formation of water- and organic-soluble metabolites, five bronchial explants (approximately 10–20 μg of mucosal DNA) metabolized BzaP at a rate similar to 2×10^6 x-irradiated Syrian golden hamster embryo cells (refs. 1 and 18; unpublished data). However, mutagenicity mediated by x-irradiated hamster embryo cells is approximately 10 times higher than that found with bronchial explants (1, 18) (Table 3). This may be due to differences in metabolic patterns in human bronchus and

hamster cells. The closer proximity of a monolayer of hamster cells than an explant to the V-79 indicator cells or the short half-life of mutagenic metabolites may also explain the higher efficiency of hamster cells.

The expression time is an important determinant for the recovery of mutants. The expression time used by other investigators has ranged from 42 to 96 hr (1–4, 18–23). Under our experimental conditions, the expression time for maximal mutation frequency for O^r was 48 hr (Fig. 2). Reconstruction experiments showed that the recovery of O^r mutants was 80% with an expression time of 48 hr and 0% at 72 hr. A similar decrease in the observed O^r mutation frequency has been previously observed (1, 2, 24, 25). We have found that this decrease correlates with the V-79 cells reaching confluence at 72 hr under our conditions. When these confluent cells are dispersed and seeded at 10^5 cells per plate and then 1 mM ouabain is added 48 hr later, the O^r mutants can be recovered in the nonconfluent culture; this is shown by the O^r mutation frequency once again reaching the peak value. The possibility of a metabolic interaction—e.g., wild-type cells draining K^+ from O^r mutants via gap junctions between the two confluent cell types—has been discussed (24). Therefore, the observed frequencies of mutation and the actual frequencies are similar with an expression time of 48 hr under our experimental conditions.

The metabolic activation of chemical carcinogens into promutagens and mutagens can now be studied directly with differentiated epithelial cells in human tissues. Human tissue-mediated mutagenesis assays may also supplement short-term bioassays (26, 27) that are important prescreens for the carcinogenicity of chemicals. Because most environmental procarcinogens require metabolic activation to ultimate carcinogens and because animal species differ in their capability to activate these procarcinogens enzymatically (28), bioassays with human target epithelial cells have obvious potential value. Finally, human tissue-mediated mutagenesis assays may be useful in testing the hypothesis that people may differ in their oncogenic and mutagenic susceptibility to environmental chemicals, depending on each individual's metabolic capability to activate and to deactivate chemical procarcinogens.

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